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HISTOCHEMICAL OBSERVATIONS ON THE LOCALIZATION OF SOME ENZYMES ASSOCIATED WITH DIGESTION IN FOUR SPECIES OF BRAZILIAN NEMERTEANS

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The processes of digestion have been investigated histochemically for several nemertean species (Jennings, 1962a; Gibson and Jennings, 1969; Jennings and Gibson, 1969; Gibson, 1970), and results so far obtained suggest that although the fundamental digestive sequence is essentially similar for all the species, differences in details can be related either to their systematic position or mode of life.

Irrespective of the nature of the food utilized, digestion occurs in two distinct phases. Initial extracellular digestion is accomplished in the intestinal lumen at an acidic pH and involves mainly proteolytic enzymes secreted by the gastrodermis. Food particles are subsequently engulfed by lamellar outgrowths of the distal ciliated cell walls (Jennings, 1969) and food vacuoles passed back into the gastrodermis for the second, intracellular, stage in digestion. This involves exopeptidases (arylamidases), acid and alkaline phosphatases and, in some species at least, carbohydrases and lipases.

The most uniform patterns of digestion are found in anoplan nemerteans (Jennings, 1962a; Jennings and Gibson, 1969). Acidophilic gland cells in the foregut, rich in carbonic anhydrase, discharge their contents during ingestion which serve both to kill prey taken alive and provide the correct lumenar pH for extracellular digestion. Mucoid secretions, discharged from other foregut glands, lubricate the food as it is passed into the intestine. Food entering the intestinal lumen stimulates the gastrodermal gland cells to discharge endopeptidases, which are responsible for early proteolysis and function optimally under acidic conditions.

Intracellular digestion initially involves endopeptidases phagocytosed along with food particles and is marked by a sharp increase in acid phosphatase activity in and around food vacuoles. Acid phosphatases may be concerned in some way with the maintenance of the correct pH for intralumenar endopeptic activity (Jennings and Gibson, 1969) or with food vacuole formation (Rosenbaum and Rolon, 1960). Slinger and Gibson (1974) demonstrating biochemically that these enzymes function optimally in the range pH 4.1–5.0, depending upon the species.

A few hours after the commencement of phagocytosis acid phosphatase and endopeptidase activity in the food vacuoles and surrounding cytoplasm declines, being replaced by alkaline phosphatases and exopeptidases. This final, alkaline, phase in digestion operates within the pH range 8.7–10.1 (Slinger and Gibson, 1974), and continues until digestion has been completed.

Endopeptidase enzymes can be demonstrated in the gastrodermal gland cells at all times, irrespective of the nutritive state, but exopeptidase activity can only be visualized histochemically in gut cells at the appropriate stage in digestion.

This contrasts markedly with blood system exopeptidases, which can be detected at all times (Gibson and Jennings, 1967).

Far greater variation in digestive physiology is found amongst the Enopla. At least two distinct types of foregut physiology have been demonstrated, one resembling the anoplan pattern and involving carbonic anhydrase production (*Prostoma*), the other achieving an intralumenar acidic pH via some other mechanism and not possessing demonstrable carbonic anhydrase (*Amphiporus, Paranemertes, Tetrastemma*) (Jennings and Gibson, 1969; Gibson, 1970).

Differences between the two classes are also found in the gastrodermal physiology; no enoplan species has yet been recorded with endopeptidase enzymes located in its gastrodermal gland cells, the enzymes instead being synthesized in and secreted from spherical inclusions housed within the columnar cells. The nature of the enzymes secreted by hoplonemertean gastrodermal glands has not yet been determined, but it is supposed that since the animals are carnivorous the secretions are proteolytic in form. Other enzymes involved in digestion essentially follow the pattern outlined for the Anopla.

A major departure from the usual type of digestive physiology is found in the bdellonemertean *Malacobdella* (Gibson and Jennings, 1969), where both morphological and physiological characters of the gut are considerably altered. These modifications, however, such as the replacement of endopeptidases by α-amylase-like carbohydrases as the principal enzymic group in digestion, and the total absence from the gastrodermis of demonstrable exopeptidases, can be entirely related to the species' atypical way of life and unselective microphagous feeding habits.

In the present study species of nemerteans belonging to families not previously investigated have been examined to determine whether or not existing known patterns of digestive physiology are evident, or whether additional variations could be detected.

Materials and Methods

The species of nemerteans investigated, listed systematically, were:

ANOPLA

Order: HETERONEMERTEA

Bascodiscus delineatus (Delle Chiaje)

ENOPLA

Order: HOPLONEMERTEA

Ototyphlonemertes affinis Kirsteuer, Ms. name
Ototyphlonemertes erneba Corrêa
Ototyphlonemertes lactea Corrêa

Ototyphlonemertes affinis is soon to be described as a new species by Dr. Ernst Kirsteuer, The American Museum of Natural History, New York (Morphology, taxonomy and ecology of the nemertean genus *Ototyphlonemertes,* with special reference to the American species—in preparation).

Specimens of *Baseodiscus* were collected from beneath stones and boulders in the intertidal zones of shores at Ubatuba, São Sebastião and Praia de Siriuba, on the coast of São Paulo State, Brazil. The three *Ototyphlonemertes* species, all psammobiontic or interstitial forms (Kirsteuer, 1967, 1971), were obtained from a sandy beach at Ilhabela on the Island of São Sebastião, some 100 km east of Santos, Brazil. Samples of sand were "panned" in the manner employed by Corrêa (1958), fresh fish meat being used to bait the sand surface for about ten minutes before panning was carried out.

The living worms were maintained in the laboratory in frequently changed, cool sea water. A selection of associated fauna and artificial foods was tested in attempts to set up a feeding series, but under laboratory conditions none of the species were ever observed to feed. Results, therefore, are based upon histological and histochemical evidence obtained from different animals fixed at progressive time intervals after collection. In the case of the *Ototyphlonemertes* species, although responding to the first bait and often showing signs of having fed on it, a definite feeding series could not be established because there was no way of knowing when the animals' last meal had been prior to accepting the bait.

Histological observations were made on paraffin wax (56° C mp) sections of specimens fixed in marine Bouin and stained either by Mallory's trichrome or the 1% aqueous Alcian blue methods.

The nature and location of enzymes was investigated in animals fixed for 2–4 hr at 4° C in 10% buffered formalin, pH 7.0, washed in chilled distilled water and frozen-sectioned at 10–12 μ on an International Equipment Co. Microtome-Cryostat Model CTF. Sections were air-dried on clean slides and rinsed in cold absolute acetone before incubation for enzyme visualization.

The following methods were used to investigate enzymes present: the Hausler (1958) technique for carbonic anhydrase; the indoxyl acetate (Holt, 1958) and α-naphthyl acetate (Gomori, 1952) methods for non-specific esterases; the Burstone and Folk (1956) L-leucyl-β-naphthylamide method for exopeptidases (arylamidases); the Burstone (1958) azo-dye method for acid phosphatases, with naphthyl AS-TR phosphate as substrate and Red-violet LB salt as simultaneous coupler; the Gomori (1952) Tween method for lipases; and the Gomori (1939) calcium salt method for alkaline phosphatases, with sodium β-glycerophosphate as substrate.

Controls for these histochemical methods included the use of heat inactivated sections and incubation media from which the specific substrates were omitted.

In addition, fat deposits were studied in paraffin wax sections of animals fixed in Flemming's osmium tetroxide fluid, and the occurrence of glycogen reserves investigated in sections after fixation in 90% alcohol containing 1% picric acid and stained by the Best's carmine method.

Observations

ANOPLA

Order: HETERONEMERTEA

Baseodiscus delineatus

Structure of the gut. The gut is divisible into two principal regions, the foregut and intestine, both of which are lined by an epithelium formed from glandular and ciliated columnar cells. By far the shorter of the two alimentary regions, the foregut can be differentiated from the intestine by its mucus-secreting gland cells and shorter, more densely arranged, cilia.

The mouth, only 1 mm or less in length, opens into a buccal cavity with walls which are deeply folded into longitudinal ridges. These ridges have been reported from other *Baseodiscus* and heteronemertean species and are interpreted as permitting the dilation of the buccal cavity for the ingestion of a large-sized meal (Jennings and Gibson, 1969; Gibson, 1974). The buccal and foregut epithelium are histologically identical but differ in their thickness. Where buccal folding is most prominent the epithelium may be up to 300–350 μ tall whereas further back in the foregut it decreases to a height of only 200–250 μ. Cilia of the columnar cells in both buccal cavity and foregut are densely arranged and 6–8 μ long.

There appear to be several types of gland cells in the anterior gut epithelium, although two major varieties can be distinguished. These are: (1) Elongate or pyriform acidophilic glands filled with numerous closely packed minute spheres 1 μ or less in diameter. Other, similarly shaped, glands with more loosely arranged contents and less obvious acidophilic affinities, may represent gland cells of the same type but in a different physiological state. The glands are negative to Alcian blue; (2) Irregularly-shaped gland cells filled with a coarsely granular cytoplasm, approximately twice as numerous as the acidophils. Many of these glands stain with the Alcian blue method for mucopolysaccharides, the intensity of staining varying from faint to deep.

Large numbers of gland cells of both groups can also be found in the parenchyma underlying the foregut and buccal epithelium, discharging their contents through the gut wall into the lumen. A similar situation has been found in lineid heteronemerteans (Jennings, 1960, 1962a; Jennings and Gibson, 1969).

The intestine is typically anoplan in form and for most of its length bears serially repeated lateral diverticula. The intestinal epithelium, or gastrodermis, is identical in both the main canal and the lateral diverticula. It consists of ciliated columnar cells, up to 150 μ tall and 6–8 μ wide and with sparsely distributed cilia 10–12 μ long, interspersed with rather smaller pyriform gland cells containing oval or spherical acidophilic globules which show a strong positive reaction to methods for the demonstration of esterases (Fig. 1). The gland cells lie proximally in the gastrodermis, tracts of discharging globules reaching up to the intestinal lumen between the columnar cells.

Towards the posterior of the body there is a gradual reduction both in the size and number of lateral diverticula and in the gastrodermal height. Immediately before the anus the columnar cells are no more than about 50–60 μ tall.

The density of gastrodermal gland cells similarly decreases posteriorly and they are completely absent from the short "rectal" region, 3.5 mm long in an animal 27 cm in length. The anus opens at the extreme posterior tip of the body.

Enzymes of the gut. Carbonic anhydrase activity has been previously reported from the foregut acidophil glands of both palaeo- and heteronemerteans (Jennings, 1962a; Jennings and Gibson, 1969), where it is believed to be concerned in the initiation of the correct intralumenar pH for the subsequent extracellular phases of proteolysis. No evidence of carbonic anhydrase activity could be demonstrated in the foregut or any other tissue in *Bascodiscus delineatus.*

The Hausler method does, however, stain many elongate ovoid bodies located in the epidermis. These bodies, which are equally strongly stained in heat-denatured control sections, are of similar size and shape to the epidermal rhabdite-like cells and the reaction in them may be due to some calcium or other salt component.

Intense esterase activity, demonstrable with both the α-naphthyl acetate and indoxyl acetate techniques, was consistently found in some 10–12% of the buccal and foregut acidophilic gland cells (Fig. 2). Esterases have not previously been recorded from these sites in any nemertean species.

In the intestine the pyriform gastrodermal glands at all times stain strongly for esterase activity (Fig. 1). Although it was not possible to utilize selective inhibitors and activators in conjunction with the indoxyl acetate method, as employed by Hess and Pearse (1958), it seems likely that the gland cell activity represents that of cathepsin C-type proteases, as recorded from other anoplan forms (Jennings, 1962a; Jennings and Gibson, 1969). There is no reason to suppose that intestinal endopeptidases are absent from this species, and both histochemical methods employed are known to react positively at sites of endopeptic activity in other nemerteans.

Sections of animals prepared eight or more hours after collection showed that numerous food vacuoles and their surrounding columnar cell cytoplasm stained positively for esterase activity. The intensity of the colored reaction product varied between specimens; in some examples only a weak activity could be seen, confined mainly to the proximal regions of the columnar cells, but in others the activity was distributed throughout the gastrodermis and appeared very much stronger.

Arylamidases (exopeptidases) of the "leucine aminopeptidase" type were visualized in vacuolar and cytoplasmic regions of the gastrodermal columnar cells in only some of the specimens investigated (Fig. 3). When present the reaction, in general, appeared to be more intense in the posterior half of the intestine. Maximum arylamidase activity in food vacuoles occurred in animals with a weak vacuolar esterase reaction. Nemertean gastrodermal arylamidases are known to be histochemically demonstrable only during the appropriate phase in the digestive sequence and recent work by Slinger (1974) has shown that whole-animal aryl-amidase extracts function optimally on the alkaline side of neutrality.

In the gut acid phosphatase activity was confined to the intestinal columnar cells, in and around food vacuoles (Fig. 4). Its degree of activity and intracellular distribution closely parallelled that shown by the gastrodermal esterases.

Alkaline phosphatase activity in the intestine (Fig. 5) is similar to that of arylamidases, its maximum visualization in cytoplasm and food vacuoles occurring at the same time. The link between these two enzyme groups has already been clearly established in nemertean worms (Jennings, 1962a; Jennings and Gibson, 1969; Gibson, 1970), and both succeed esterase and acid phosphatase demonstration in the digestive sequence.

At times when arylamidase activity can not be shown in the gut, alkaline phosphatases are confined to the distal border of the gastrodermis, forming a narrow but intensely staining zone of activity a few microns deep. The occurrence of alkaline phosphatases at this site, where they are believed to be independent of the nutritive state, has been found in other heteronemertean species (Jennings, 1962a; Jennings and Gibson, 1969).

The Tween method for the demonstration of lipases has not generally proved to be sensitive enough with nemertean tissues, unless the animals are maintained on a high fat content diet (Jennings, 1962a). In some of the *Baseodiscus* specimens studied a faint but definite brownish reaction, absent from control slides, could with prolonged incubation be distinguished in several food vacuoles. This activity, occurring at the same time as peak arylamidase visualization, may represent the occurrence of lipolytic enzymes.

No other enzymes were demonstrated in any part of the alimentary system.

Food reserves. Fat deposits are principally restricted to the distal half of the gastrodermal columnar cells, occurring as droplets of varying diameter from 5–6 μ downwards. Glycogen, appearing as small granules scattered throughout the gastrodermis, can also be found in lesser quantities in freshly collected animals.

Enzymes of other tissues. Several enzymes were demonstrated at sites of activity other than the gut.

Arylamidase activity was consistently found in the blood system endothelium, although the intensity of the staining reaction varied from weak to strongly positive depending upon the part of the body and individual animal. The reaction was generally strongest in the lateral blood vessels where they ran alongside the intestine. Blood system arylamidases are well known from nemerteans (Gibson and Jennings, 1967).

Esterase activity was localized in many tissues of the body, including the epidermis (Fig. 6), ciliated cerebral canal and some parts of the body wall and proboscis musculature. At these sites the degree of activity was normally weak to moderate, although the proximal epidermal regions were often intensely stained. Consistently strong esterase activity was found in ganglionic cells of the principal nerve tracts, especially around the fibrous core of the cerebral ganglia and lateral nerve cords (Figs. 6 and 7). No instance was observed of any esterase or other enzymic activity in the fibrillar component of the nervous system, and it is probable that the esterases demonstrated are in fact of the cholinesterase type, as found in the nervous system of other nemertean species (Ling, 1969a, 1969b).

Esterase activity could, in some examples, also be localized in certain gland cells of the proboscis epithelium.

Alkaline phosphatases were irregularly found at several sites. Strong activity was seen in the nephridial ducts and cerebral canal, weak to moderate activity showed in the gland cell region of the cerebral organs. Other cells of the cerebral

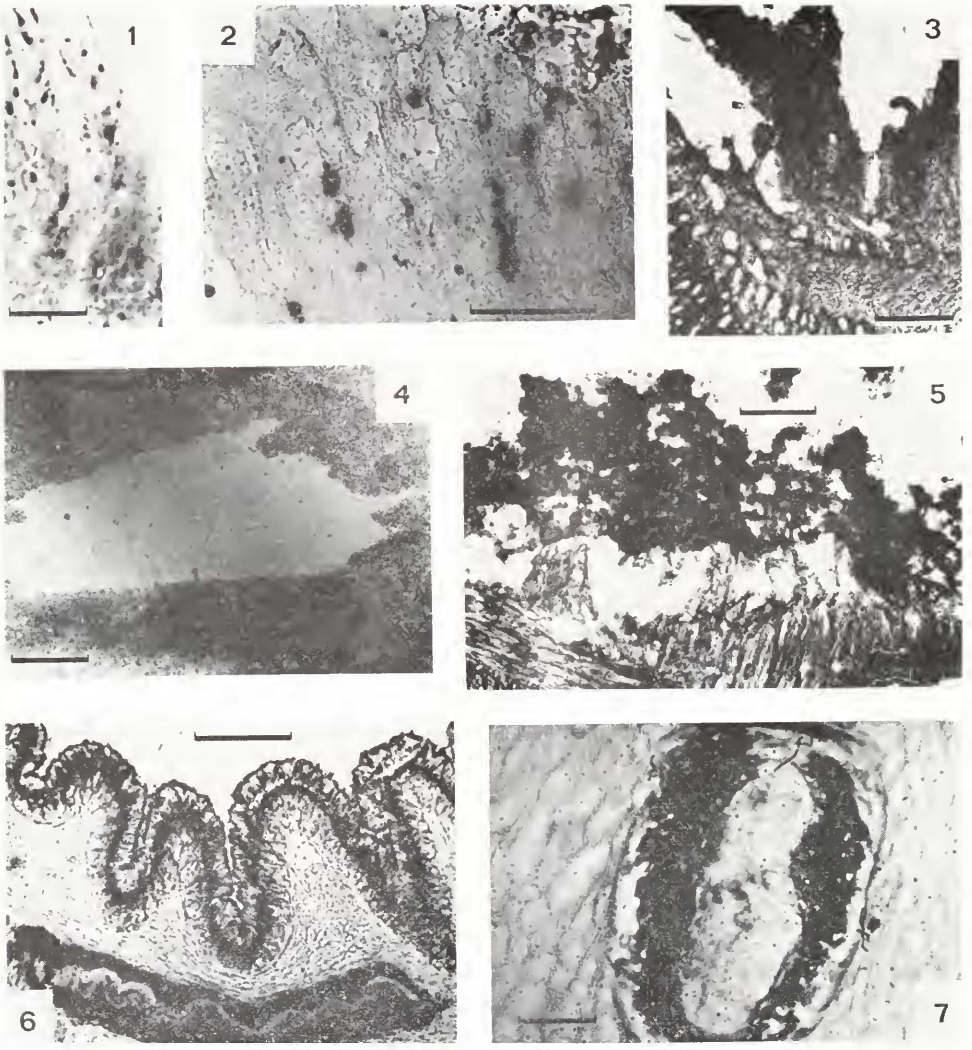


FIGURE 1. *Bascodiscus delineatus;* Longitudinal section through part of the gastrodermis to show acidophilic gland cells staining intensely with the Holt indoxyl acetate method for esterases (proteases), scale = 105 μ.

FIGURE 2. *Bascodiscus delineatus;* Longitudinal section through a portion of the foregut epithelium to show esterase activity localized in some of the acidophilic gland cells; Gomori α-naphthyl acetate method, scale = 70 μ.

FIGURE 3. *Bascodiscus delineatus;* Transverse section through a part of the gastrodermis, showing arylamidase activity (black) generally distributed throughout most of the columnar cells; Burstone and Folk method, scale = 150 μ.

FIGURE 4. *Bascodiscus delineatus;* A part of the intestine showing acid phosphatase activity distributed through the distal regions of the gastrodermis; Burstone azo-dye method, scale = 130 μ.

organs on occasion reacted faintly to the acid phosphatase technique. Traces of these enzymes were also found in the proximal regions of the epidermis, but the reaction was always extremely weak and, although absent from control slides, not positively identified.

No other sites of enzymic activity were recorded in *Baseodiscus delineatus.*

ENOPLA

Order: HOPLONEMERTEA

Ototyphlonemertes affinis, O. erneba and *O. lactea*

Structure of the gut. The gut structure of two of the three species has been described by Corrêa (1950, 1954) and all are extremely similar. In common with other monostiliferous hoplonemerteans, the rhynchocoel and buccal cavity of *Ototyphlonemertes* share a common anterior aperture, the rhynchodaeal pore. This opens into the rhynchodaeum from which the rhynchocoel leads dorsally and the oesophagus ventrally.

Three regions of the gut can be recognized histologically, the oesophagus, the stomach and intestine. In the Brazilian *Ototyphlonemertes* the intestine lacks a caecum, the stomach therefore opening directly into it without the intervention of a pyloric tube such as is found in many hoplonemerteans.

The oesophagus opens from the rhynchodaeum in front of the cerebral ganglia and extends posteriorly below the brain as a short straight ciliated tube devoid of gland cells. It opens directly into the stomach, which consists of a folded epithelium formed from densely ciliated columnar or cuboidal and large numbers of gland cells. The epithelial height differs between the species; in *O. lactea* the stomach lining is only 10–12 μ tall compared with 25 μ in *O. affinis* and 30–35 μ in *O. erneba.* Most of the gland cells contain a finely granular to fibrillar basophilic secretion, but isolated acidophilic glands filled with homogeneous contents are irregularly distributed between the basophils. Stomach columnar cell cilia in all three species are densely arranged and 4–4.5 μ long.

Posteriorly the stomach opens directly into the intestine with only a slight narrowing of its lumen, unlike the situation depicted for *O. lactea* by Corrêa (1954; Plate 7, fig. 30).

The gastrodermis is very similar in structure to that described for other hoplonemerteans, consisting of acidophilic gland cells interspersed with sparsely ciliated columnar cells. The distribution of gland cells is more or less uniform throughout the intestinal length in *O. affinis* and *O. erneba,* but in *O. lactea* the anterior half contains many more gland cells than the posterior, the ratio being approximately 3 : 1. Corrêa (1954) notes the anterior aggregation of gland cells

FIGURE 5. *Baseodiscus delineatus;* Longitudinal section through part of the intestine to show the cytoplasmic localization of alkaline phosphatase activity; Gomori calcium salt method, scale = 83 μ.

FIGURE 6. *Baseodiscus delineatus;* Longitudinal section to show intense esterase activity present in the proximal epidermal regions and ganglionic cells of a lateral nerve cord; Gomori α-naphthyl acetate method, scale = 270 μ.

FIGURE 7. *Baseodiscus delineatus;* Transverse section through a lateral nerve cord, showing esterase activity confined to the ganglionic nerve sheath; Holt indoxyl acetate method, scale = 67 μ.

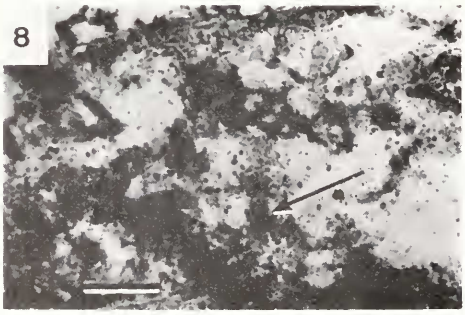
in *O. lactea,* and in this and other species calls them erythrophil glandular cells. The glands are filled with acidophilic spheres 1 μ or less in diameter, similar sized or slightly larger globules also occurring in the columnar cells, particularly in the proximal half. Columnar cell inclusions are similar in appearance to those described from other hoplonemerteans (Jennings and Gibson, 1969; Gibson, 1970) which contain endopeptidases. The cilia of the gastrodermis are 7–8 μ long, the overall gastrodermal height some 45–50 μ in all three species. Corrêa (1948) contrasts the low epithelial height of the stomach with the high gastrodermal development in other species (*O. brevis* and *O. evelinae*) without mentioning cellular dimensions. In the animals used during the present investigation the contrast between stomach and intestinal height depends upon the species concerned and is least in *O. erneba.*

As Corrêa (1950, 1954) reported, the "rectal" region of the intestine is not dilated and can be distinguished from the more anterior intestinal regions by the absence of gland cells. The region also lacks diverticula, but throughout the intestinal length these are at best only poorly developed.

Enzymes of the gut. No evidence of carbonic anhydrase activity could be found in the gut or any other tissue of the body.

Strong esterase activity was found in the gastrodermal acidophilic glands (Fig. 8), demonstrable by both histochemical methods employed. The individual globules filling the glands stained intensely in most of the animals studied, but in others could not be distinguished. Jennings (1962a) found that in the heteronemertean *Lineus ruber* gastrodermal gland cells which had discharged their spheres shortly after feeding failed to react to techniques for the visualization of cathepsin C-type endopeptidases. It thus seems probable that in those *Ototyphlonemertes* specimens with negative gland cell reactions a similar situation is prevailing, an inference supported by the fact that this was only seen in animals fixed soon after collection.

No other hoplonemertean species yet investigated has possessed histochemically demonstrable enzymes in its gastrodermal gland cells (Jennings and Gibson,



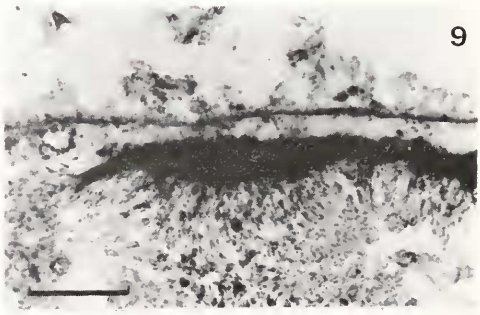


FIGURE 8. *Ototyphlonemertes affinis;* Longitudinal section through the gastrodermis to show the occurrence of esterases (proteases) in food vacuoles and gland cells. A pair of large gland cells is arrowed; Holt indoxyl acetate method, scale = 42 μ.

FIGURE 9. *Ototyphlonemertes erneba;* Longitudinal section through the anterior proboscis to show the localization of esterase activity in the basal regions of the epithelium. Some activity can also be seen in more distal parts of gland cells; Gomori α-naphthyl acetate method, scale = 67 μ.

1969; Gibson, 1970); instead endopeptidases are produced by and secreted from acidophilic globules contained in the columnar cells. In *Ototyphlonemertes* gastrodermal cytoplasmic inclusions do react positively for esterases but, in the absence of a feeding series, it cannot with certainty be determined whether these represent food vacuoles or the more usual hoplonemertean endopeptic vesicles. However, Corrêa (1950; page 218), in her histological study of the digestive processes in *O. brevis,* makes no mention of columnar cell inclusions other than after extracellularly digested material has been "absorbed" and shows as ". . . droplets, granules, and vacuoles of different size . . ." This evidence, together with the fact that esterase positive vesicles are not always visible, and that when they are there is also evidence of cytoplasmic activity around them, leads us to the conclusion that in *Ototyphlonemertes* the inclusions represent food vacuoles undergoing intracellular digestion. No other esterase activity could be distinguished in the digestive tract.

Little evidence of arylamidase activity could be found in the gut. A faint reaction, absent from control slides, appeared in the gastrodermal tissues of *O. lactea* and *O. erneba,* none being discerned in *O. affinis.* In specimens exhibiting this faint activity food vacuoles were negatively responsive to the esterase methods and, conversely, when strong vacuolar esterase activity was seen no evidence of arylamidases could be distinguished.

Acid phosphatase activity in the gut was entirely confined to gastrodermal food vacuoles and their surrounding cytoplasm, appearing most intense at the same time as maximal vacuolar esterase activity. When evidence of intracellular esterase function was missing, no acid phosphatases could be demonstrated in the gut.

Varied reactions to the calcium salt method for alkaline phosphatases were obtained. In the gastrodermis intense cytoplasmic and vacuolar activity was seen in the same specimens exhibiting weak esterase and acid phosphatase reactions. At such times the anterior half of the intestine showed a stronger reaction than the posterior, confined mostly to the proximal parts of the columnar cells. Other individuals, and especially those showing some evidence of weak arylamidase activity, possessed alkaline phosphatases throughout their gastrodermal length. The staining intensity was greatest in these animals, appearing equally strong in both food vacuoles and cytoplasm.

As with *Bascodiscus,* prolonged incubation in the Tween medium irregularly resulted in a brownish vacuolar deposit, absent from control sections, appearing in many of the gastrodermal food vacuoles. This reaction may indicate the occurrence of intracellularly acting lipases, apparently effective at the same time as both esterases and arylamidases. No evidence of other sites of enzymatic activity could be found in the gut.

Food reserves. The principal food reserves are fat droplets, filling the intestinal columnar cells in freshly collected animals. They range from minute globules, less than 1 μ in diameter, up to amorphous masses 3–4 μ across. Lesser amounts of fat deposits can also be found in the body wall muscle layers and in the anterior proboscis epithelium. Glycogen, as small particulate deposits, is confined to the gastrodermis, where it is mainly restricted to the distal half of the columnar cells.

Enzymes of other tissues. Enzymic activity was found in several other sites within the three species investigated.

Strong arylamidase activity is present in the blood vascular system, mostly confined to the endothelial layers of the vessels but, on occasion, also showing within the blood fluid. The occurrence of these enzymes in nemertean blood systems seems likely to be universal.

Arylamidases were also found at two other sites. In *O. affinis* a weak reaction was sometimes seen in parts of the body wall musculature, and in *O. erneba* whole mount preparations resulted in a darkly staining band of activity extending across the stylet bulb just posterior to the central proboscis stylet.

Esterolytic activity appeared widespread in several body tissues, occurring strongly in all three species in the proximal epidermal regions, less intensely as a thin distal epidermal band, in the basal parts of many gland cells in the anterior proboscis epithelium (Fig. 9), and in ganglionic cells surrounding the lateral nerve cords. At these sites enzymic activity was consistently demonstrable by both the α-naphthyl acetate and indoxyl acetate methods, and appeared to be independent of the nutritive state. In *O. affinis* incubation in the indoxyl acetate medium gave a faint positive reaction in parts of the body wall musculature; it is possible that this activity can be related to the arylamidases demonstrated at the same site in this species.

In general acid phosphatases at sites other than the gut parallel the distribution of esterases, particularly in the epidermis and proboscis epithelium. The proximal epidermal regions especially give a strong positive reaction to the Burstone naphthol phosphate technique.

Alkaline phosphatases were weakly evident in the proximal epidermis of all three species and, in *O. affinis*, also showed in some regions of the principal musculature. A strong reaction for these enzymes was obtained in the posterior proboscis epithelium, and a weak or negative response was seen forming a thin distal band of activity in the epithelium of the anterior proboscis.

Discussion

Gibson (1972) comments that nemertean worms are, for the most part, carnivorous or scavenging macrophages. The *Ototyphlonemertes* species, although in the present investigation consistently refusing to accept either whole or squashed naturally associated psammobiontic polychaetes, turbellarians or other macroscopic fauna under laboratory conditions, clearly possess scavenging habits since they respond so rapidly to the fish meat baiting method of collection. They do, in fact, show evidence of wide dietary preferences; Corrêa (1948) comments that *Ototyphlonemertes* do not apparently live exclusively by scavenging and cites an example of *O. brevis* with a recently eaten crustacean in its gut. Subsequently, Corrêa (1950) used *O. brevis* for feeding experiments in the laboratory, using as food polychaetes, crustaceans and portions of striated muscle from *Armadillidium vulgare*.

No identifiable food remains were found in any of the *Baseodiscus* specimens studied but, as judged from the structure of the gut and the nature of its associated enzymic complement, this species too will be found to be carnivorous and/or scavenging in its habits.

Previous studies on nemertean digestive physiology (Jennings, 1960, 1962a; Gibson and Jennings, 1969; Jennings and Gibson, 1969; Gibson, 1970) have demonstrated that, although the basic sequence in the digestion of food material is the same for all species, interspecific differences in the nature and location of enzymes can be detected which may be related to systematic position, diet and mode of life. The evidence gleaned from the current histochemical studies shows that in all four species the intestinal gland cells obviously synthesize and secrete enzymes for initial extracellular digestion, the subsequent stages being carried out intracellularly in food vacuoles and involving other enzymes of cytoplasmic origin. Since lysosomes appear to be invariably present in animal cells and are known to contain a whole range of hydrolytic enzymes optimally active under acidic conditions (Duve, 1967), it seems reasonable to suppose that intracellular digestion is at least partly due to lysosomal enzymes. Future ultrastructural studies are needed to provide more detailed information about enzyme localization in nemerteans.

The specific differences seen between the species require some further consideration. The absence of demonstrable carbonic anhydrase activity from the foregut of *Baseodiscus delineatus* is unusual, and has not been reported before for anoplan nemerteans. Similarly, the occurrence of esterase activity in many of the foregut glands in this species has not been found in any other nemertean, and it is tempting to suggest that the two features are in some way related. It is clear that during the ingestive phase both mucus and esterase producing glands in the foregut discharge their contents. In anoplan nemerteans foregut secretions are involved in killing live prey, in establishing the appropriate intralumenar pH for subsequent extracellular proteolysis, and in facilitating swallowing by the lubricative action of mucoid products. Initiation of suitable lumenar conditions for the early stages of intestinal digestion must, in *Baseodiscus*, be achieved by a mechanism that does not involve carbonic anhydrase enzymes, as found in several hoplonemertean species including *Ototyphlonemertes*. The role of the esterase secretions in the foregut may in some way be concerned in this mechanism; a more plausible explanation of the part played by these enzymes, however, is that the animals possess carnivorous feeding habits similar to those found in many triclad turbellarians, and that the enzymes are used in the penetration of the prey's body wall and early disruption of its body contents. Pharyngeal gland cells secreting endopeptidase enzymes for this purpose are found, for example, in *Polycelis* and *Orthodemus* (Jennings, 1959, 1962b). An extracorporeal histolytic phase, essential to the feeding mechanism, has been described for the hoplonemerteans *Amphiporus lactifloreus* (Jennings and Gibson, 1969) and *Nipponnemertes pulcher* (Berg, 1972), although in these species the secretions responsible for the breakdown of prey tissues are believed to be derived from the proboscis rather than the stomach.

The remaining stages of digestion in *Baseodiscus* follow the usual heteronemertean sequence. Gastrodermal gland cells discharge enzymes for the extracellular breakdown of food material, which is then phagocytosed by the columnar cells and completely digested intracellularly in food vacuoles. Early intracellular digestion involves esterases and acid phosphatases, the final stages utilize arylamidases and alkaline phosphatases. In the gastrodermis the esterases observed

probably represent endopeptidase activity for, although appropriate activators and inhibitors were not available for use in the study, there is no logical reason to suppose that enzymes of other groups are synthesized by the gland cells. With the exception of the unselective microphage, *Malacobdella,* in which the intestinal gland cells apparently secrete carbohydrases (Gibson and Jennings, 1969), all nemerteans depend primarily upon endopeptic enzymes for extracellular proteolysis, including those hoplonemerteans in which the precise nature of the gland secretions has not been established.

Ototyphlonemertes brevis alone amongst the species investigated from this genus is known to be actively carnivorous (Corrêa, 1948, 1950) but, from a comparison of their complement of digestive enzymes and closely similar gut morphology, there is every reason to suppose that the present species possess at least related feeding habits.

Mucus and carbonic anhydrase were not demonstrated in any part of the gut, and in this respect *Ototyphlonemertes* parallels other marine enoplans (Gibson and Jennings, 1969; Jennings and Gibson, 1969; Gibson, 1970). Only in the freshwater hoplonemertean *Prostoma rubrum* has carbonic anhydrase activity been located in the foregut. In other forms the intense basophilia shown by so many of the stomach glands, as found in all three *Ototyphlonemertes* species, is taken to indicate that a major proportion of the stomach secretions are acidic in nature.

The demonstration of esterase activity in the gastrodermal gland cells has not before been reported for any hoplonemertean species. Corrêa (1950) reported that immediately after food was taken into the intestine the epithelium became thinner, interpreting this phenomenon as a symptom of secretion although it might also be explained as due to stretching of the gut wall for the accommodation of the food. Her assumption was supported by evidence of extracellular digestion and, since the animals are carnivorous, the esterase activity seen in the present species must be indicative of endopeptic proteolysis. This conclusion is supported by Corrêa's experiments involving the rapid liquefaction of crustacean muscle fragments by artificially freed gland cell contents. Further, her studies strongly suggest that the enzymes are normally present in the gland cells in an active state and do not require unmasking by foregut secretions before they can be effective.

The apparent absence from the gastrodermis of endopeptidase-secreting columnar cell vesicles, previously believed to be a typical hoplonemertean character, is not altogther surprising when the gland cells themselves contain enzymes of this group. No nemertean species has yet been shown to possess both sites of endopeptic synthesis in its gastrodermis, and the earlier interpretation that columnar cell inclusions represent food vacuoles undergoing early intracellular digestion seems justified. After the phagocytosis of food material, intracellular digestion appears to follow the usual nemertean sequence and involves the normal complement of enzymes. The small amount of evidence for arylamidase activity, and its absence from *O. affinis,* may be simply due to the fact that animals were not studied at the appropriate digestive phase. These enzymes, in the gastrodermis, cannot at other times be visualized histochemically. If, in fact, *Ototyphlonemertes* usually ingests a large-sized meal (*e.g.,* see Corrêa, 1950, Plate IV, f. 16–17, 19–20) it might be reasonably expected that the duration of digestion is accordingly prolonged.

The patterns of digestion in the four Brazilian species thus show features not found in previous studies, although they fit perfectly into the broad concept of digestion in this phylum. *Bascodiscus* differs from other heteronemerteans in its possession of esterase enzymes in and absence of carbonic anhydrase from the foregut, physiological modifications which may be related to its possible feeding habits. The *Ototyphlonemertes* species, on the other hand, fall intermediate between known patterns of digestion in that their foregut is typically hoplonemertean in form and function, whereas their intestinal physiology is like that found in anoplan nemerteans. These differences indicate that very many more species need to be studied before an overall picture of digestive physiology in the phylum as a whole can be built up.

The food reserves in the four Brazilian species studied appear to show no features not previously found in other free-living forms. Fat deposits form the principal reserve store, with glycogen accumulation seemingly playing only a supportive role.

Of the enzymes found at sites other than the gut arylamidases are consistently present in the blood vascular system. They are here presumably involved in the circulation of peptides about the body, as fully discussed by Gibson and Jennings (1967).

Esterase activity was also found in all species located in the epidermis, ganglionic cells of the nervous system and proboscis epithelium. Weak alkaline phosphatase activity too occurred in the epidermis, with additionally acid phosphatases appearing at this site and in association with proboscis epithelial esterases in the *Ototyphlonemertes* species. Epidermal enzymic activity is likely to be concerned either in the absorption of nutrients from the environment, a mechanism initially proposed by Fisher and Cramer (1967) but subscribed to by Jennings and Gibson (1969), or in the secretion of mucoid and other substances which may play some defensive role against potential predators, as outlined by Gibson (1972). *Bascodiscus* in particular, when handled, discharges copious amounts of a thick and viscid mucus.

Esterases seen in the ganglionic cells of the major nerves and, in *Bascodiscus*, around the brain, are likely to represent cholinesterase activity related to the passage of nerve impulses. Kamemoto (1957) found evidence of extremely high cholinesterase activity in *Prostoma rubrum* compared with that of the marine *Cerebratulus lacteus*, and variations in the localization of these enzymes in different species may depend upon individual physiology. Cholinesterases are also believed to be involved in some aspects of muscle physiology (summarized by Gibson, 1972), and esterases found in the body wall and proboscis musculature are probably concerned with this component of the body metabolism. The significance of the weak arylamidase activity found in the body wall musculature of *O. affinis* may similarly be related to muscular function.

Alkaline phosphatases are known to be present in the nephridial ducts of certain nemerteans (Danielli and Pantin, 1950; Jennings and Gibson, 1969), where they are believed to be involved in some way with the modification of nephridial fluid composition. These enzymes in *Bascodiscus* may be expected to possess a related function.

The various enzymes observed at other sites in the different species are not so easily explained. A relationship between alkaline phosphatase and esterase in the ciliated cerebral canal and cerebral organs of *Baseodiscus* may be suggested. There is some evidence in nemerteans that components of the cerebral organs, as well as other tissues, may be involved in some endocrine function, and the occurrence of neurosecretion is well known in lineid heteronemerteans (Lechenault, 1962, 1963, 1965; Bianchi, 1969a, 1969b). It is thus possible that in *Baseodiscus* the cerebral canal and organ enzymes are concerned in endocrine physiology, perhaps through a chemotactic mechanism. Recent studies on the ultrastructure of the cerebral organs of *Lineus ruber* have shown that they may be chemoreceptive, mechanoreceptive or photoreceptive in function (Ling, 1969a, 1969b, 1970), although it is not known with certainly what precise function the organs have. The finding of enzymes at these sites in *Baseodiscus* may, therefore, point towards the cerebral organs possessing a chemoreceptive rather than any other function. This suggestion can, however, be no more than conjecture at this time.

Esterases found in the proboscis epithelium in the four species are likely to be related to some aspect of proboscis secretion, and also is the alkaline phosphatase activity occurring in the posterior proboscis epithelium of the *Ototyphlonemertes* species.

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Summary

1. A comparative histochemical study has been made of the nature and location of enzymes associated with digestion in four species of Brazilian nemerteans.

2. The basic digestive physiology is similar in the four species (Heteronemertea: *Baseodiscus delineatus;* Hoplonemertea: *Ototyphlonemertes affinis, O. erneba, O. lactea*), with extracellular acidic proteolysis being followed by phagocytosis and the completion of digestion intracellularly by proteases and lipases acting synchronously. Intracellular digestion is initially acidic and then alkaline, with acid and alkaline phosphatases being associated with the appropriate phase.

3. The proteolytic enzymes responsible for extracellular digestion are produced within the gastrodermal gland cells in all four species. The occurrence of histo-

chemically demonstrable proteolytic enzymes has not before been found in hoplonemertean gastrodermal gland cells. In *Ototyphlonemertes* the glands are physiologically similar to those of anoplan species and, accordingly, the usual hoplonemertean columnar cell endopeptic vesicles are apparently missing.

4. *Baseodiscus delineatus* does not possess demonstrable carbonic anhydrase activity in its foregut, a feature not previously recorded from anoplan nemerteans. In this respect it resembles certain hoplonemertean species.

5. A proportion of the foregut acidophil glands in *Baseodiscus delineatus* contain esterolytic enzymes. These enzymes have not been found in the foregut of any other nemertean species. It is suggested that their occurrence in *Baseodiscus* may indicate an extracorporeal preingestive stage in digestion.

6. The food reserves consist mainly of fat, located in the gastrodermis in all species, but there are supplementary deposits of glycogen at the same site.

7. The occurrence of enzymic activity at locations other than the gut is recorded.

8. These findings are discussed in relation to previous work on the digestive and other physiological processes of nemertean worms.

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